Report

Current Biology

EXO70I Is Required for Development of a Sub-domain of the Periarbuscular Membrane during Arbuscular Mycorrhizal Symbiosis

Highlights

- Arbuscule branching is impaired in *Medicago truncatula exo70i* mutants
- Incorporation of STR and STR2 into the periarbuscular membrane is limited in *exo70i*
- EXO70I accumulates adjacent to the tips of the arbuscule branches.
- EXO70I partially co-localizes with Vapyrin and interacts with Vapyrin.

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In Brief

Zhang et al. demonstrate that EXO70I is required to support arbuscule development during AM symbiosis. EXO70I is located in discrete zones adjacent to arbuscule branch tips where it interacts with Vapyrin. The data provide insights into development of the periarbuscular membrane during AM symbiosis.

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EXO70I Is Required for Development of a Sub-domain of the Periarbuscular Membrane during Arbuscular Mycorrhizal Symbiosis

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SUMMARY

In eukaryotic cells, polarized secretion mediated by exocytotic fusion of membrane vesicles with the plasma membrane is essential for spatially restricted expansion of the plasma membrane and for the delivery of molecules to specific locations at the membrane and/or cell surface. The EXOCYST complex is central to this process, and in yeast, regulation of the EXO70 subunit influences exocytosis and cargo specificity [1, 2]. In contrast to yeast and mammalian cells, plants have upwards of 23 EXO70 genes with largely unknown roles [3–6]. During arbuscular mycorrhizal (AM) symbiosis, deposition of the plant periarbuscular membrane (PAM) around the fungal arbuscule creates an intracellular membrane interface between the symbionts. The PAM has two major membrane sub-domains, and symbiosis-specific transporter proteins are localized in the branch domain [7–11]. Currently, the mechanisms and cellular machinery involved in biogenesis of the PAM are largely unknown. Here, we identify an EXO70I protein present exclusively in plants forming AM symbiosis. *Medicago truncatula exo70i* mutants are unable to support normal arbuscule development, and incorporation of two PAM-resident ABC transporters, STR and STR2, is limited. During arbuscule branching, EXO70I is located in spatially restricted zones adjacent to the PAM around the arbuscule hyphal tips where it interacts with Vapyrin [12–14], a plant-specific protein required for arbuscule development. We conclude that EXO70I provides a specific exocytotic capacity necessary for development of the main functional sub-domain of the PAM. Furthermore, in contrast to other eukaryotes, plant EXO70s have evolved distinct specificities and interaction partners to fulfill their specialized secretory requirements.

RESULTS AND DISCUSSION

EXO70I Is Induced during AM Symbiosis and Co-expressed with Vapyrin

Most flowering plants have the capacity to form symbiotic associations with arbuscular mycorrhizal (AM) fungi and benefit from phosphate delivered by their fungal symbionts [15]. During AM symbiosis, the AM fungus colonizes the root cortical cells where it develops branched hyphae called arbuscules that function in nutrient exchange with the plant. Arbuscules are housed in an apoplastic compartment surrounded by the periarbuscular membrane (PAM), which is generated by the root cell coincident with arbuscule development. The PAM closely follows the contours of the arbuscule, and distinct sub-domains of the PAM, the branch and trunk domains, can be defined physically and also by their resident proteins; essential symbiotic phosphate transporters MtPT4/OsPT11 [7, 8, 16–18] and ABC transporters STR and STR2 [10, 11] are present exclusively in the branch domain. Polar localization of MtPT4 requires expression coincident with development of the branch domain of the PAM and is predicted to involve reorientation of the default secretory pathway [7, 9, 16]; however, our understanding of the cellular machinery involved in development of the PAM is limited. The location of exocytotic marker proteins [19] and the requirement for VAMP721/722 [20] indicate the involvement of an exocytotic pathway. Additionally, Vapyrin/PAM1, a protein of unknown function, may be part of the cellular machinery for development of the PAM [12–14].

Through a transcriptional co-expression analysis, we identified Medtr1g017910, a member of the EXO70 gene family that is co-expressed with Vapyrin. Medtr1g017910 is a member of the EXO70I cluster [6] (Figure S1), which occurs only in plants that form AM symbiosis, a phylogenetic distribution that is shared by Vapyrin [12] and several other genes essential for symbiosis [11, 16]. *EXO70I* transcript levels increase 3- to 24-fold during AM symbiosis and correlate with colonization of the root system (Figure 1A). The *EXO70I* promoter is active at low levels in mock-inoculated roots, mostly in the root tips (Figures S2A and S2B), and is highly active in cortical cells containing arbuscules (Figure 1B).

exo70i Mutants Are Unable to Support Arbuscule Development

A *Medicago truncatula* line, NF4919 (*exo70i-1*), with a Tnt1 transposon insertion located 1,962 nucleotides downstream of the predicted *EXO70I* start codon was obtained (Figure S2C and Supplemental Experimental Procedures) and its symbiotic phenotype assessed. At 2, 4, 6, and 8 days post contact with *G. versiforme* spores, the numbers of hyphopodia, cortical infections, and infections with arbuscules in *exo70i-1* did not differ significantly from wild-type (Figures S2D and S2E). Thus, the fungal hyphae traverse the epidermis and also enter cortical cells

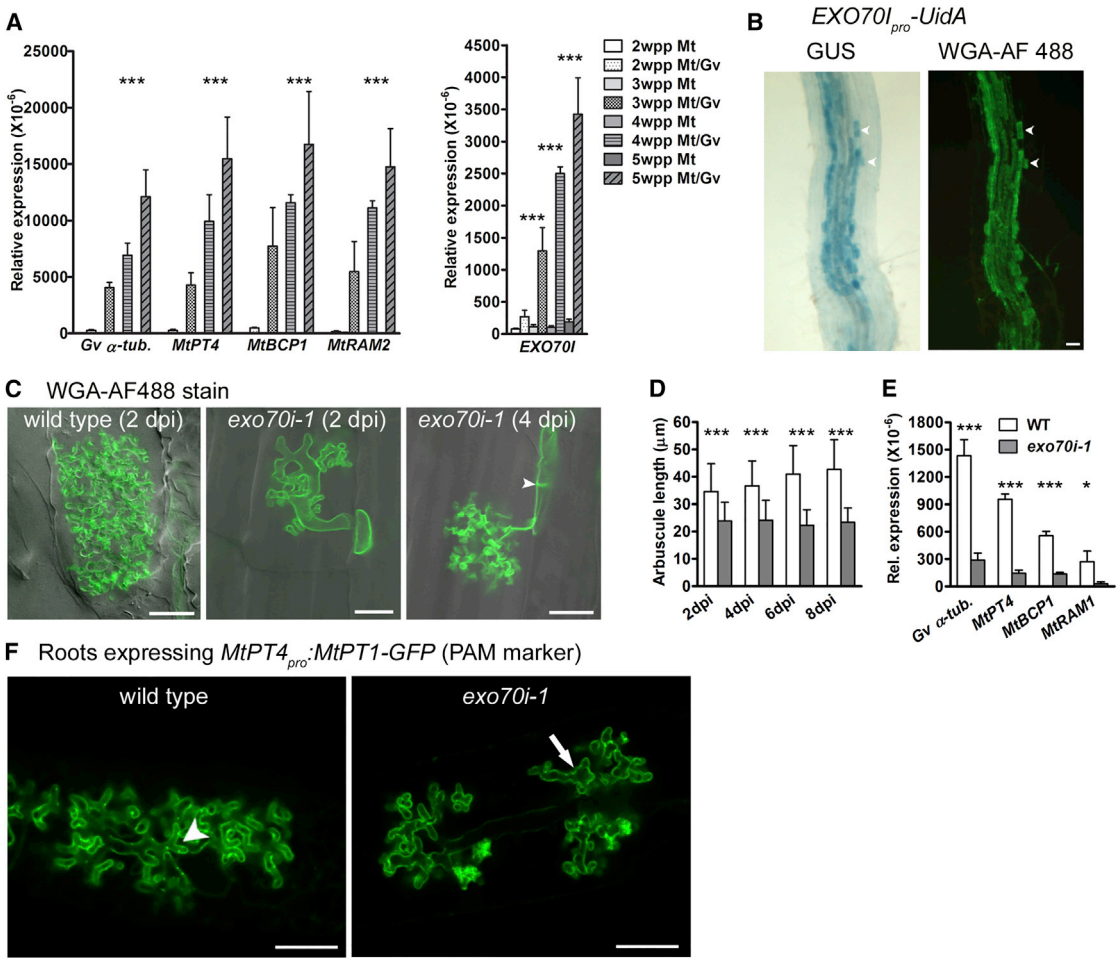

Figure 1. *EXO70I* Is Induced during AM Symbiosis and Required to Enable Arbuscule Development

(A) Relative expression of *G. versiforme* α-*tubulin*, *MtPT4*, *MtBCP1*, *MtRAM2* and *EXO70I* in non-colonized *M. truncatula* A17 roots (Mt) and roots colonized with *G. versiforme* (Mt/Gv) at 2, 3, 4, and 5 weeks post planting (wpp) into inoculum. *G. versiforme* α-*tubulin* transcripts provide an indication of the level of fungal RNA in the mycorrhizal root samples. *MtPT4*, *MtBCP1*, and *MtRAM2* serve as markers of arbuscule development and of colonization of the root system. Data are averages ± SEM (n = 3, where n denotes the number of independent root samples). Significant differences between control and inoculated roots (***$p \leq 0.001$).

(B) An *M. truncatula* mycorrhizal root expressing $EXO70I_{pro}$:*UidA* stained for GUS (left panel) and stained with WGA-Alexafluor 488 (right panel) to reveal the fungus. GUS and WGA-Alexafluor 488 staining overlap. Arrowheads indicate arbuscules. The scale bar represents 40 μm.

(C) Arbuscules in wild-type at 2 days post inoculation (dpi) and *exo70i-1* at 2 dpi and 4 dpi. Overlay images of the green fluorescent signals from WGA-Alexafluor 488 staining of the fungal cell walls and the corresponding differential interference contrast (DIC) images. Arrowhead points to a septum. Images are single optical sections. Scale bars represent 10 μm.

(D) Arbuscules in *exo70i-1* roots colonized with *G. versiforme* are significantly shorter in length than those in a wild-type segregant at 2, 4, 6, and 8 dpi. Data are averages ± SD (***$p < 0.0001$, Student's t test, $n > 35$).

(E) Relative expression of *G. versiforme* α-*tubulin*, *MtPT4*, *MtBCP1*, and *MtRAM2* in *exo70i-1* and wild-type segregant roots colonized with *G. versiforme* at 21 days post planting (dpp) into inoculum. *G. versiforme* α-tubulin transcripts provide an indication of the level of fungal RNA in the mycorrhizal root samples. *MtPT4*, *MtBCP1*, and *MtRAM2* serve as markers of arbuscule development and colonization of the root system. Data are averages ± SD (n = 4, where n denotes the number of independent root samples). Significant differences between control and inoculated roots (*$p \leq 0.05$, ***$p \leq 0.001$).

(F) Wild-type and *exo70i-1* roots expressing $MtPT4_{pro}$:*MtPT1-GFP*, which results in labeling of the PAM. The PAM surrounds the arbuscule, thus revealing its shape. Roots are colonized with *G. versiforme*. In wild-type, the arrowhead points to a dichotomous hyphal branch. In *exo70i-1*, the arrow indicates an example of aberrant hyphal branching with swollen hyphae. Scale bars represent 10 μm. Images are single optical sections.

in *exo70i-1* as efficiently as in wild-type roots. However, arbuscules in *exo70i-1* showed a major defect in development relative to those in wild-type; arbuscule trunks developed normally, but arbuscule branching was reduced, and hyphal branches were aberrant and frequently distorted in shape (Figure 1C). This inability to develop was followed by premature formation of septa and arbuscule collapse, and consequently, arbuscules in *exo70i-1* are significantly smaller than those in wild-type roots (Figures 1D and S2F). Consistent with a defect in arbuscule development, transcript levels of genes that serve as markers of arbuscule development were reduced significantly in *exo70i-1* relative to wild-type (Figure 1E). This aberrant arbuscule development phenotype was observed in three additional independent *exo70i* alleles obtained subsequently to *exo70i-1*

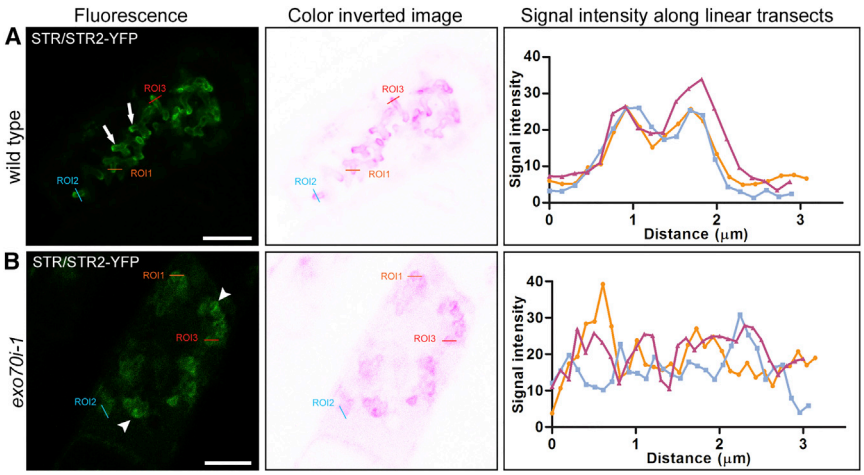

Figure 2. Limited Incorporation of PAM-Resident ABC Transporters, STR and STR2, into the PAM in *exo70i-1* Roots

Confocal microscopy images (single optical sections) of *M. truncatula* root cortical cells containing arbuscules. Left panels: fluorescence images. Middle panels: color-inverted images. Right panels: graphs showing signal intensity along the three linear transects (regions of interest [ROIs] 1, 2, 3) shown in the images.

(A) Wild-type roots expressing $STR_{pro}STR\text{-}nYFP$ and $STR2_{pro}STR2\text{-}cYFP$ (STR/STR2-YFP) [11]. Arrows indicate a sharply defined STR/STR2-YFP signal in the PAM surrounding the fine branches of the arbuscule.

(B) *exo70i-1* roots expressing $STR_{pro}STR\text{-}nYFP$ and $STR2_{pro}STR2\text{-}cYFP$ (STR/STR2-YFP). Arrowheads indicate diffuse STR/STR2-YFP signals around the arbuscule branches. Additionally, diffuse YFP signals are present throughout the cell.

Roots were colonized with *G. versiforme*. Scale bars represent 10 μm. All images are single optical sections. In the signal intensity graphs (right panels), the two peaks and steep shoulders on the transects across arbuscule branches in wild-type roots are consistent with signal from protein in the PAM. In contrast, transects through arbuscule branches in *exo70i-1* roots show a broad erratic signal with many small peaks, suggesting no specific point of accumulation. Single representative images are shown for each genotype with data from three transects. However, for each genotype, data were obtained from 51 linear transects through arbuscule branches from nine independent cells (images). The number of peaks by genotype was analyzed using a generalized linear mixed model with a Poisson distribution and log link with a random effect of image. The mean peak numbers in wild-type and *exo70i* were 2.38 and 7, respectively. This difference is statistically significant ($F_{32.2,1} = 67.87$, $p < 0.0001$).

(Figures S2C and S2F–S2I). Data from the *M. truncatula* gene expression atlas (http://mtgea.noble.org/v3/) indicate that *EXO70I* transcript levels also increase during root nodule symbiosis with rhizobium bacteria. However, *exo70i-1* plants inoculated with *S. meliloti* developed root nodules similar in number and morphology to those on wild-type roots (Figures S2J–S2S), indicating that *EXO70I* is not essential for rhizobial symbiosis. Likewise, *EXO70I* is not required for primary root growth (Figures S2T–S2V).

The classical function of the EXOCYST complex is to tether post-golgi exocytotic vesicles to the target plasma membrane prior to fusion mediated by SNARE proteins [21]. Consequently, we predicted a role for EXO70I in biogenesis of the PAM. To determine whether root cells in *exo70i* establish a membrane around the aberrant arbuscules, we used an integral membrane protein-GFP marker (*MtPT1-GFP*) expressed from the MtPT4 promoter [9] to visualize the PAM. In wild-type root cells, a sharply defined GFP signal, characteristic of MtPT1-GFP [9], outlined the arbuscules and revealed the typical dichotomously branched hyphae with hyphal tips that were uniform in shape and size (Figure 1F). Likewise, in *exo70i-1* cells, the MtPT1-GFP signal also outlined the arbuscules indicating that the fungus is enveloped in a membrane (Figure 1F). Live imaging of *exo70i-1* cells expressing MtPT1-GFP further clarified the arbuscule development phenotype, revealing arbuscules with short, random hyphal branches that were uneven in width and frequently swollen at the tip (Figure 1F).

A Periarbuscular Membrane Surrounds Arbuscules in *exo70i-1*, but Branch Domain-Specific ABC Transporters, STR and STR2, Are Not Efficiently Incorporated into the Membrane

Since a PAM is present in *exo70i-1*-colonized cells, we questioned whether EXO70I might be involved in secretion of PAM-resident proteins. In yeast, differential secretion of cargoes occurs via regulation of EXOCYST components [22, 23]; however, in plants, it has been speculated that individual EXO70 proteins might provide unique docking specificities [3, 24]. To investigate a potential involvement of EXO70I in polar secretion of the branch domain-specific ABC transporters STR and STR2, we introduced split-YFP-tagged versions of the STR and STR2 proteins into *exo70i* (Figure 2). In contrast to wild-type, where a sharply defined STR/STR2-YFP fluorescent signal was visible around the fine branches of the young arbuscules (Figure 2A), in *exo70i-1*, the signals around the arbuscule branch tips were diffuse, and hazy signals were apparent throughout the cell (Figure 2B). Color-inverted images (Figures 2A and 2B, middle panels) and graphs of signal intensity (Figures 2A and 2B, right panels) provide additional visualization and analyses of these differences. In wild-type roots, the signal intensities along linear transects across arbuscule branches showed a distinct pattern with two prominent peaks, flanked by steep shoulders (Figure 2, right panels). This pattern is consistent with signals arising from the PAM. In contrast, in *exo70i-1* roots, signal intensities along the linear transects showed numerous smaller peaks, suggesting limited accumulation in the PAM. To provide statistical support for a difference in the signal intensity patterns in wild-type and *exo70i*, 51 linear transects across arbuscule branches from nine independent cells in each genotype were analyzed for peak number. There was a significant effect of genotype on peak number ($F_{32.2,1} = 67.87$, $p < 0.0001$). Based on these data, we infer that STR and STR2 are not incorporated efficiently into the PAM in *exo70i-1*. The diffuse signals associated with the aberrant arbuscule branches could potentially arise from untethered exocytotic vesicles accumulating in the vicinity of the hyphae. Similar observations have been made in a yeast exocytosis mutant during budding [23, 25]. Reduced accumulation of STR and STR2 in the PAM could explain part of the

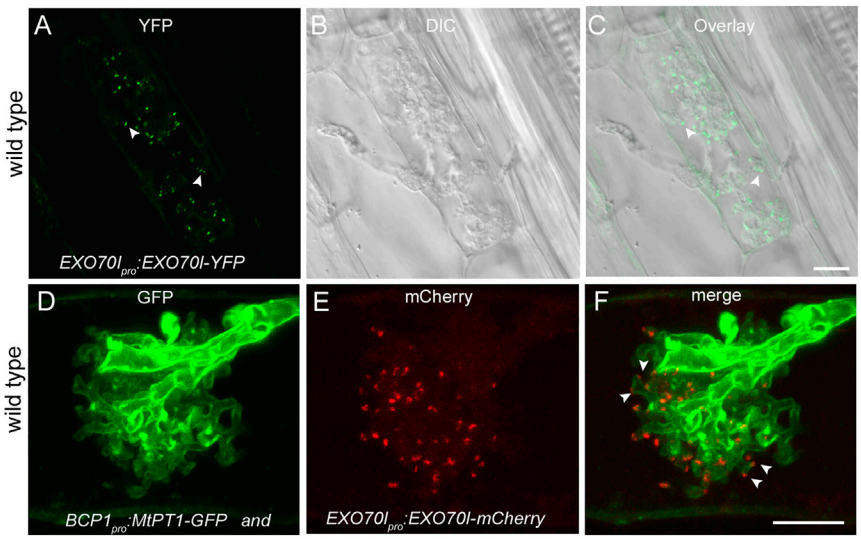

Figure 3. EXO70I Accumulates Adjacent to the Hyphal Tips of Arbuscules in *M. truncatula* Roots

(A–C) Confocal microscopy images of live *M. truncatula* roots expressing *EXO70I$_{pro}$:EXO70I-YFP* colonized with *G. versiforme*. Fluorescence image (A), corresponding DIC image (B), and corresponding overlay image (C) of a cortical cell containing arbuscule. Images are single optical sections.

(D–F) Confocal microscopy images of live *M. truncatula* roots co-expressing a membrane marker *BCP1$_{pro}$:MtPT1-GFP* and *EXO70I$_{pro}$: EXO70I-mCherry* colonized with *G. versiforme*.

(D) Green fluorescence from MtPT1-GFP marking the PAM.

(E) Red fluorescence from EXO70I-mCherry.

(F) Merged green and red channels.

Arrowheads point to subcellular localization of EXO70I adjacent to the hyphal tips. Scale bars represent 10 μm. Images are projections of 27 optical sections on the Z axis taken at 0.13-μm intervals.

exo70i phenotype as arbuscule development fails in *str* mutants [10, 11]. Currently, it is unclear whether incorporation of other PAM-resident proteins is limited in *exo70i*; however, the arbuscule phenotype of *exo70i* is not an exact replica of the *str* phenotype, which might suggest a broader effect on protein secretion.

Thus, the arbuscule morphology in *exo70i* mutants coupled with the limited incorporation of STR and STR2 into the PAM indicate that EXO70I activity is critical during the early branching phase of arbuscule development.

EXO70I Accumulates in Spatially Restricted Zones Adjacent to the PAM at Arbuscule Branch Tips

Studies of the EXOCYST in yeast suggest that there are two pools of EXO70 protein, one of which is located on the target membrane along with the SEC3 subunit, while the other is located on the exocytotic vesicles along with the other EXOCYST subunits [26]. Thus, the location of EXO70 is indicative of active exocytosis and can also be predictive of the site of vesicle tethering [21]. In contrast, in *C. albicans*, where hyphal tip growth is much faster, EXO70 is found only on the target membrane where it appears as a distinct crescent at the hyphal tip [27]. In plants, EXO70s have been localized to the plasma membrane [4, 28, 29] and developing cell plate [30] where a classical role in exocytosis is predicted, but they are also associated with a variety of subcellular compartments [3, 31, 32] including autophagic vesicles [33] where they may have acquired new roles [24].

To determine the sub-cellular location of EXO70I, we generated *EXO70I-YFP* fusions expressed from the native promoter, demonstrated their functionality via complementation of *exo70i-1* (Figure S3A), and then examined their locations in wild-type root cells during symbiosis. Fluorescent signals from both fusions were visible as punctate spots or occasionally crescents within colonized cells (Figures 3A–3C and S3B–S3D). In some cases, a diffuse signal was also present in the cytoplasm. When overlaid with differential interference contrast (DIC) images that showed the arbuscule branches, EXO70-YFP signals appeared to be located at the hyphal tips of fine arbuscule branches. In cells containing collapsed arbuscules, the punctate signals were absent, but the diffuse fluorescent signal was present in the cytoplasm (Figure S3E). To enable simultaneous visualization of the PAM and EXO70I, roots were transformed with a single transfer DNA (T-DNA) containing a PAM marker (*BCP1$_{pro}$:MtPT1-GFP*) and *EXO70I$_{pro}$: EXO70I-mCherry*. In cells with developing arbuscules, discrete patches of mCherry fluorescence were visible adjacent to the arbuscule hyphal tips (Figures 3D–3F and S4A–S4C). Hyphal tips in the initial stage of dichotomous branching showed distinct punctate signals associated with both of the emerging branches (Figures 3F and S4C). These localized EXO70I-mCherry signals were only associated with the fine hyphal branches and were not seen near hyphal trunks or thick branches (Figure S3E). This localization pattern is consistent with the defects in development of fine arbuscule branches observed in *exo70i* mutants and with the apparent reduction in incorporation of STR/STR2-YFP into the PAM. Previous studies indicated that when expressed in mycorrhizal carrot roots, an *Arabidopsis* EXO84-GFP fusion localized to domes around the hyphae, suggesting that other EXOCYST components may also be recruited to this zone [19].

Although EXO70I-mCherry signals were present close to the hyphal tips, they did not co-localize with the GFP signal from the PAM marker (Figures S4D and S4E), which suggests that the EXO70I-mCherry signals do not arise from protein attached to the membrane but rather adjacent to it. The patches of mCherry signal are too large to represent single exocytotic vesicles but could perhaps represent clusters of vesicles, possibly functionally analogous to the spitzenkörper [34] or alternatively a reservoir from which EXO70 is cycled and recycled to the PAM to maintain a high rate of vesicle tethering.

Vapyrin Shows Interaction and Partial Co-localization with EXO70I

Vapyrin is a protein of unknown function required for arbuscule formation [12–14]. It is comprised of an N-terminal vesicle-associated protein (VAP) domain and a C-terminal ankyrin-repeat

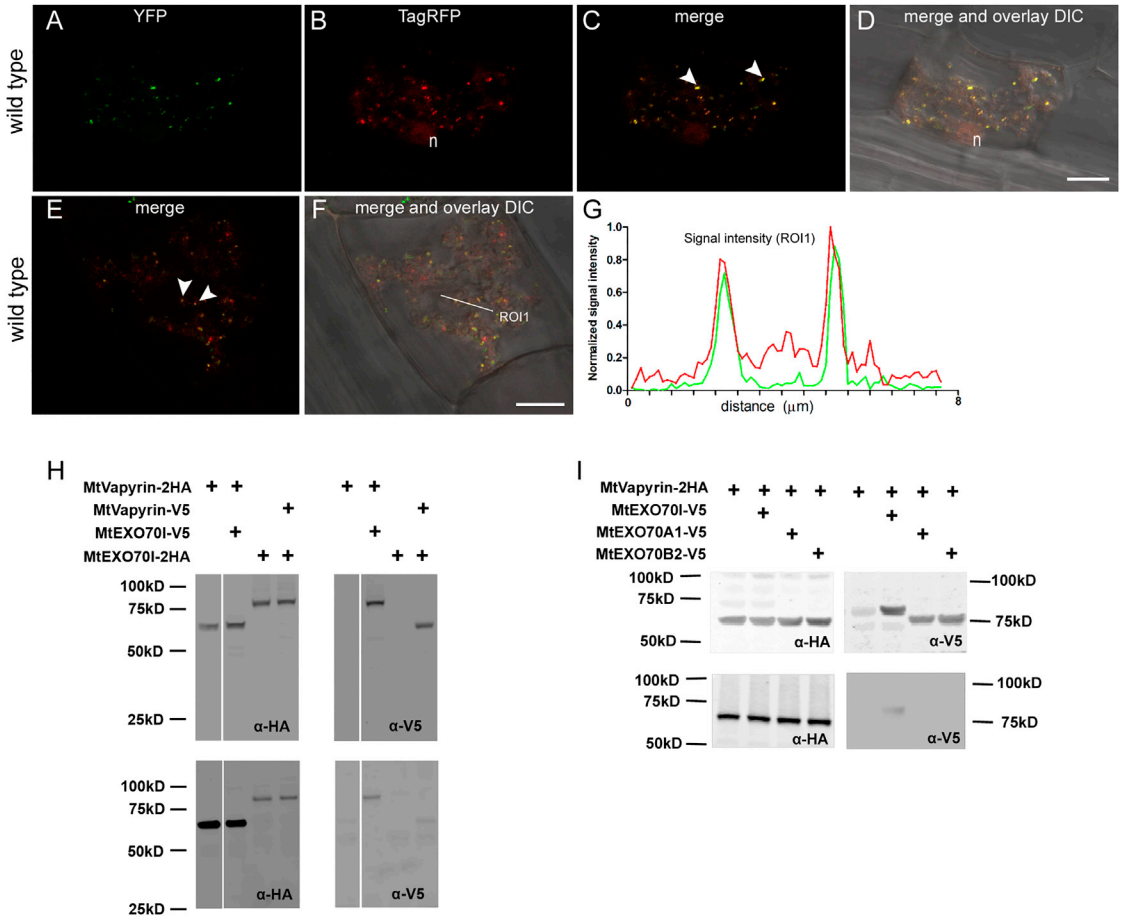

Figure 4. Co-localization and Interaction of EXO70I and Vapyrin

(A–G) Confocal microscopy images of two cells containing arbuscules from wild-type *M. truncatula* roots expressing *EXO70I$_{pro}$:EXO70I-YFP* and *Vapyrin$_{pro}$:Vapyrin-TagRFP*.

(A) EXO70I-YFP green, punctate signals.

(B) Vapyrin-TagRFP, red punctate signals. and signals in the cytoplasm and nucleus (n).

(C) Merged YFP and TagRFP channels indicate partial co-localization of YFP and TagRFP signals (yellow) as indicated by arrowheads; Pearson correlation coefficient = 0.76.

(D) An overlay of fluorescence with the corresponding DIC image. The scale bar represents 15 μm.

(E–G) A second cell containing an arbuscule.

(E) Merged YFP and TagRFP channels indicate partial co-localization of YFP and TagRFP punctate signals (yellow) as indicated by arrowheads.

(F) An overlay of fluorescence with the corresponding DIC image.

(G) Signal intensities in the YFP (green line) and RFP (red line) channels along the linear ROI shown in (F) show co-occurrence of signal intensity peaks corresponding to two punctate signals visible in (E) and (F). The single-channel images are shown in Figure S5.

Scale bars represent 10 μm. Images are single optical sections.

(H) Co-immunoprecipitation analysis shows that Vapyrin interacts with EXO70I. *35S$_{pro}$: Vapyrin-2HA* and *35S$_{pro}$:EXO70I-V5* were co-expressed in *N. benthamiana* leaves. Additionally, *35S$_{pro}$:Vapyrin-V5* and *35S$_{pro}$:EXO70I-2HA* were co-expressed in *N. benthamiana* leaves. The upper panels show immunoblots of total protein extracts from leaf discs (input protein) probed with the anti-HA and anti-V5 antibodies. The lower panels show immunoblots following immunoprecipitation with agarose beads conjugated with anti-HA antibody. The blots were probed with anti-HA and anti-V5 antibodies. The experiments demonstrate that Vapyrin interacts with EXO70I.

(I) Co-immunoprecipitation analysis shows that Vapyrin interacts with EXO70I, but not with two other members of the EXO70 family, EXO70A1 (Medtr1g090620.1) or EXO70B2 (Medtr3g031130.1). *35S$_{pro}$:Vapyrin-2HA* was co-expressed with either *35S$_{pro}$:EXO70I-V5*, *35S$_{pro}$:EXO70A1-V5* or *35S$_{pro}$:EXO70B2-V5* in *N. benthamiana* leaves. The upper panels show immunoblots of total protein extracts from leaf discs (input protein) probed with the anti-HA and anti-V5 antibodies. The lower panels show immunoblots following immunoprecipitation with agarose beads conjugated with anti-HA antibody. The blots were probed with anti-HA and anti-V5 antibodies. The experiment demonstrates that Vapyrin interacts with EXO70I, but not with EXO70A1 or EXO70B2.

domain, and as both domains are predicted to be involved in protein-protein interactions, a potential role as a scaffold protein has been proposed [12–14]. Previously, Vapyrin-GFP signals were observed in mobile and static puncta in the cytoplasm, and weaker diffuse signals were associated with the nucleus [12, 13]. The punctate signals were observed only in colonized cells. Co-expression of *EXO70-YFP* and *Vapyrin-TagRFP* each from their native promoters revealed partial co-localization of

the two proteins and a subset of the discrete EXO70-YFP signals at the hyphal tips co-localized with Vapyrin-TagRFP static, punctate signals. Vapyrin-TagRFP and EXO70-YFP punctate signals that were not co-localized were also apparent (Figures 4A–4G and S5A–S5F), and additionally, only the Vapyrin signal was visible in the nucleus (Figures 4B and 4D). Initial attempts to localize Vapyrin in *exo70i* were unsuccessful, possibly because AM-induced gene expression is low in *exo70i* (Figure 1E).

To evaluate a possible interaction between Vapyrin and EXO70I, reciprocal co-immunoprecipitation experiments were carried out in *Nicotiana benthamiana* cells. These revealed that Vapyrin and EXO70I can physically interact (Figure 4H). In contrast, Vapyrin did not interact with two other EXO70 proteins selected at random from the EXO70 A and B clusters (Figure S1), indicating a level of specificity to the Vapyrin-EXO70I interaction (Figure 4I).

Conclusions

The initial discovery of the expansion of the EXO70 family in plants inspired much speculation about specialization of secretory activities in plant cells and potential novel functions for EXO70s [24]. Here, we identify an EXO70 that is essential for AM symbiosis and reveal a level of functional specialization that extends to a specific membrane sub-domain. Based on the spatially restricted location of EXO70I and phenotype of *exo70i*, we conclude that EXO70I is required for development of the branch domain of the PAM during AM symbiosis. This domain is the major functional domain of this symbiotic membrane, and limited incorporation of two essential ABC transporters into the membrane could partially explain the arbuscule development defect in *exo70i* mutants; however, this doesn't explain the aberrant hyphal growth. Whether reduced deposition of the PAM would influence hyphal morphology and branching is unclear, and it is possible that an aspect of signaling is also impaired in *exo70i*. Currently, signals that trigger dichotomous branching of the hyphae or that drive secretion of the PAM are unknown, but EXO70I could potentially enable focal secretion of a signal into the periarbuscular apoplast or specific placement of a receptor in the PAM. In *Arabidopsis*, EXO70B1 controls PAMP (pathogen-associated molecular pattern) signaling potentially through recycling signaling proteins [35].

The *exo70i* phenotype indicates that EXO70I is not essential for developing the trunk domain of the PAM, and consequently, other EXO70s must fulfill these initial secretory requirements. The *M. truncatula* gene expression atlas (http://mtgea.noble.org/v3/) indicates that *EXO70I* is one of several EXO70 genes expressed in cortical cells containing arbuscules, and transcript levels of two other EXO70s, Medtr2g096230.1 and Medtr5g073450.1, exceed those of *EXO70I*. Consequently, development of the complete PAM probably requires the concerted activities of multiple EXO70 proteins.

The discrete location of EXO70I, adjacent to the PAM at the hyphal tips, is striking, and additional studies are required to determine whether these signals are associated with endosomal compartments or other sub-cellular structures. A key question is how this spatially restricted location is established. We provide evidence for interaction and partial co-localization with Vapyrin and speculate that Vapyrin may serve as a scaffold protein that recruits or maintains EXO70I adjacent to the PAM at the hyphal tips. Just as EXO70s are more numerous in plants relative to yeast and mammals, so the mechanisms of their recruitment to specific locations within the cell may differ, possibly involving proteins such as Vapyrin, which exist only in the plant kingdom.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.06.075.

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